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Reports

Highly parallel gene-to-BAC addressing using microarrays

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Second-generation sequencing now provides the potential for low-cost generation of whole-genome sequences. However, for large-genome organisms with high repetitive DNA content, genome-wide short read sequence assembly is currently impossible, with accurate ordering and localization of genes still relying heavily on integration with physical and genetic maps. To facilitate this process, we have used Agilent microarrays to simultaneously address thousands of gene sequences to individual BAC clones and contiguous sequences that form part of an emerging physical map of the large and currently unsequenced 5.3-Gb barley genome. The approach represents a cost-effective, highly parallel alternative to traditional addressing methods. By coupling the gene-to-BAC address data with gene-based molecular markers, thousands of BACs can be anchored directly to the genetic map, thereby generating a framework for orientating and ordering genes, and providing direct links to phenotypic traits.

Physical map-based genome sequencing strategies have recently been superseded by relatively low-cost but ‘quick and dirty’ second-generation sequencing (2GS) based on whole-genome shotgun approaches (1,2). However, for genomes that comprise a high proportion of repetitive DNA and gene families that have evolved by duplication, de novo assembly of 2GS data remains problematic, at best providing information restricted to low copy regions (3). While this type of output has considerable and immediate utility, it remains intrinsically limited through a lack of context, encompassing little information about gene location or order. Current 2GS draft assemblies of large and complex genomes are therefore a temporary substitute for a gold standard reference sequence which remains the primary goal of most research communities.

Based on current technology, such a reference sequence can still realistically only be delivered by robust but expensive physical map-based genome sequencing (4). This is typically centered on high information content fingerprinting (HICF) of bacterial artificial chromosome (BAC) clones that are assembled into contiguous sequences (contigs) prior to sequencing the Minimal

Tiling Path (MTP) of clones (5,6). The physical map overcomes issues associated with large recombinationally inert regions typical of large and repetitive genomes, and provides the necessary context for exploring genome structure and organization, both within and among species. A key step in the physical map-based sequencing strategy is anchoring individual BAC clones and contigs to high-resolution genetic maps. This provides both a framework for orientating and ordering contigs, as well as a direct connection to mapped phenotypic traits (4). However, currently established anchoring techniques tend to be low-throughput or involve many processing steps (7).

We describe a novel application of DNA microarray technology to potentially anchor thousands of genes to thousands of BACs. The work was conducted on the large [5.3 Gb (8)] unsequenced barley genome but is generally applicable to any organism for which BAC resources and genetic maps exist.

Materials and methods

Barley BAC library pooling strategy

Genomic DNA from barley cultivar Morex was used to construct a BAC library (HVVMRXALLcA) in *pIndigoBac536*.

The original library contained 147,840 BAC clones (in 385 384-well plates) with an average insert size of 125 kb, giving a total of $\sim 3.7\times$ barley genome coverage. The BAC library was divided by the commercial vendor Amplicon Express (Pullman, WA, USA) into 55 sequential Super Pools (SPs), each consisting of seven 384-well plates. For each SP, BAC DNA was initially pooled into seven sets corresponding to each of the 384-well plates, 16 sets corresponding to each of the 384-well plate rows of all seven plates, and 24 sets corresponding to each of the 384-well plate columns of all seven plates. The seven plate, 16 row, and 24 column sets were then further pooled to create five Matrix Plate Pools (MPPs), eight Matrix Row Pools (MRPs), and 10 Matrix Column Pools (MCPs), respectively, for a total of 23 Matrix Pools (MPs). This Matrix pooling strategy results in each BAC being contained in two different MPs for each type of MP (Plate, Row, or Column) (i.e., in two of the five MPPs, two of the eight MRPs, and two of the 10 MCPs). The strategy for pooling, hybridization and deconvolution of the BACs is summarized in Figure 1.

Amplification of pooled BAC DNA

SP and MP BAC DNAs were supplied as above. As the concentration of pooled

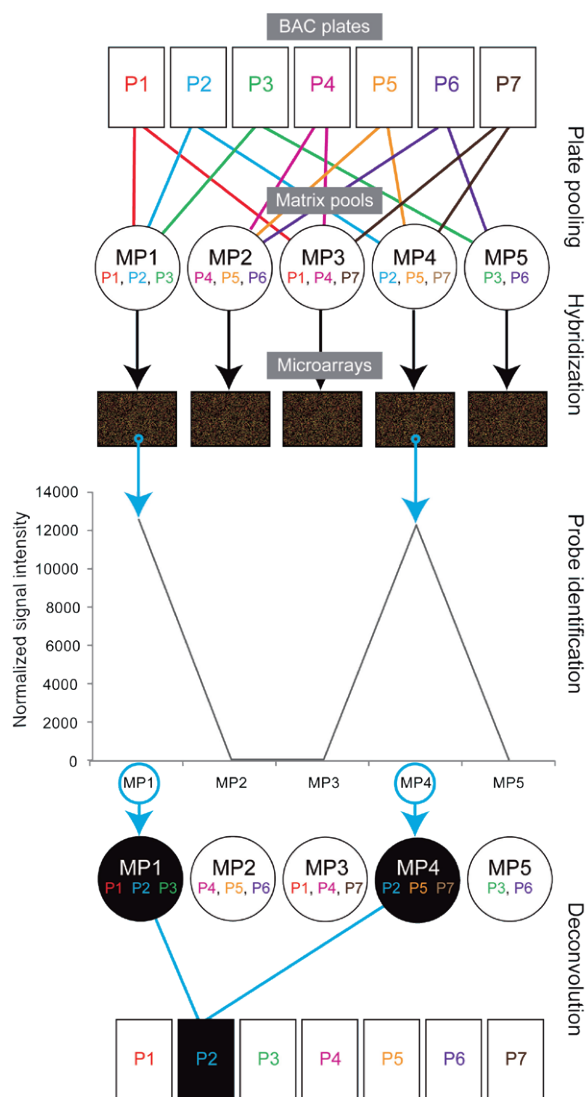


Figure 1. Schematic of BAC pooling, microarray hybridization, and deconvolution procedure. Only the strategy for processing Matrix Plate Pools (which are labelled and referred to simply as Matrix Pools in this figure) is shown, but the same principle applies to the Matrix Row and Column pools. BAC clones, each from a single plate, are initially pooled together (P1–P7), followed by a second pooling to form the Matrix Pools (MP1–MP5), such that each BAC appears in two independent MPs. Following hybridization of amplified MP DNA to microarrays, probes are identified with significant signal in two of the MPs (for single-copy genes). Deconvolution enables identification of the original plate (in this example, P2) containing a BAC clone with a gene fragment corresponding to the microarray probe sequence. This is combined with the row and column deconvolution data to determine the full original BAC addresses.

DNA supplied was very low (~5 ng/μL), high-concentration stocks were produced from each pool using the phi29 DNA polymerase rolling circle amplification method (GenomiPhi V2 DNA Polymerase Kit, GE Healthcare, Piscataway, NJ, USA). Amplification conditions were as recommended by the manufacturer: 2 μL each DNA pool was denatured in 9 μL sample buffer at 95°C for 5 min and subsequently placed on ice. A mixture of 1 μL GenomiPhi phi29 DNA polymerase and 9 μL reaction buffer, containing dNTPs and random hexamers, were added to the

denatured samples. After 2 h at 30°C, the reaction was terminated by heating for 10 min at 65°C. DNA yield and quality were checked on 0.7% agarose.

Microarray labeling and hybridization

A custom microarray SCRI_Hv35_44k_v1 was designed (design 020599; Agilent, Santa Clara, CA, USA) representing 42,302 barley expressed sequence tag (EST) contig sequences. Sequences for this design were selected from a total of 50,938 putative unigenes from HarVest assembly 35 (www.harvest-web.org) representing

~450,000 ESTs. Selection was based upon contig orientation derived from (i) homology to the nonredundant protein database (NCBI nr), (ii) homology to ESTs from directional cDNA libraries, and (iii) presence of a polyA tract. The microarray was designed with one 60-mer probe per selected unigene in 4x44k format using default parameters in the Agilent eArray software (<https://earray.chem.agilent.com/earray>). All of the probes were designed to be within or close to the 3' untranslated regions of the ESTs to maximize selection of unique probe sequences. In addition, the entire EST collection was utilized for cross reference within eArray software to minimize chances of nonspecific hybridization. Full details can be found at ArrayExpress (www.ebi.ac.uk/arrayexpress; accession no. A-MEXP-1728).

Two-channel processing of the microarrays was used, with the entire set of MP DNAs from one SP labeled entirely with Cy3 and the entire set of MP DNAs from another SP labeled with Cy5. In total, 23 arrays were utilized for the two complete sets of MPs (equivalent to two SPs) described in the text. Briefly, amplified MP DNA (200 ng) was labeled using a modified BioPrime Array CGH Genomic DNA Labeling System (Invitrogen, Carlsbad, CA, USA): amplified MP DNA in 11 μL was added to 10 μL 2.5× random primer reaction buffer mix and denatured at 95°C for 5 min prior to cooling on ice. To this was added 2.5 μL modified 10× dNTPs buffer (1.2 mM each of dATP, dGTP, dTTP; 0.6 mM dCTP; 10 mM Tris pH8.0; 1 mM EDTA), 1 μL either Cy3 or Cy5 dCTP (1 nM) and 0.5 μL Klenow enzyme (20 U) and incubated for 16 h at 37°C. Labeled samples for each array were combined and unincorporated dyes removed using Qiaquick PCR Purification Kit (Qiagen, Valencia, CA, USA) as recommended, eluting with 20 μL EB buffer (Qiagen). Hybridizations and washing were performed as recommended (Agilent Protocol v5.5). Scanning was performed with an Agilent G2505B scanner using default settings and data extracted using Agilent FE software (v9.5.3). The experimental design and all microarray data have been submitted to ArrayExpress (www.ebi.ac.uk/arrayexpress; accession no. E-TABM-1017).

Data preprocessing and deconvolution

Data were preprocessed and normalized using GeneSpring software (v7.3; Agilent). Extracted data from each of the two sets of MP arrays were imported separately and normalization performed as for single-channel arrays (data transformation

of individual probe measurements to a minimum of 0.01; per chip normalization to the 50th percentile). Data were exported from GeneSpring for downstream analysis.

Deconvolution of plate, row, and column pools was performed using scripting (Supplementary Material) in GenStat (VSN International, Hemel Hempstead, UK) by identifying maximum signal above background in two out of five MPPs, two out of eight MRPs, and two out of 10 MCPs (see strategy in Figure 1). Identification of a unigene in two MPPs, for example, was measured by the log ratio of the difference between the second- and third-largest MPP signals and the difference between the third largest and the median probe signal of the remaining MPPs. As we are expecting two significant outlying signals from each MP type (for a single copy gene), this approach identifies those pools with the first- and second-highest significant probe signals above background. This ratio was then weighted using a Gompertz function and identification confirmed when the weighted ratio (gScore) exceeded an empirically assigned threshold (0.9). A match with the BAC clone was confirmed when the combined identified unigene plates, rows and columns corresponded to the known BAC pooling addresses.

Illumina sequencing of SP1 DNA

Separate samples (five in total) of amplified SP1 DNA were sequenced on an Illumina GAII sequencer (San Diego, CA, USA), with a single lane used for each sample. This resulted in a total of 9,269,872 reads of 54-nucleotide length and 57,650,261 reads of 70-nucleotide length (4.5 Gb total). The sequence reads were quality-

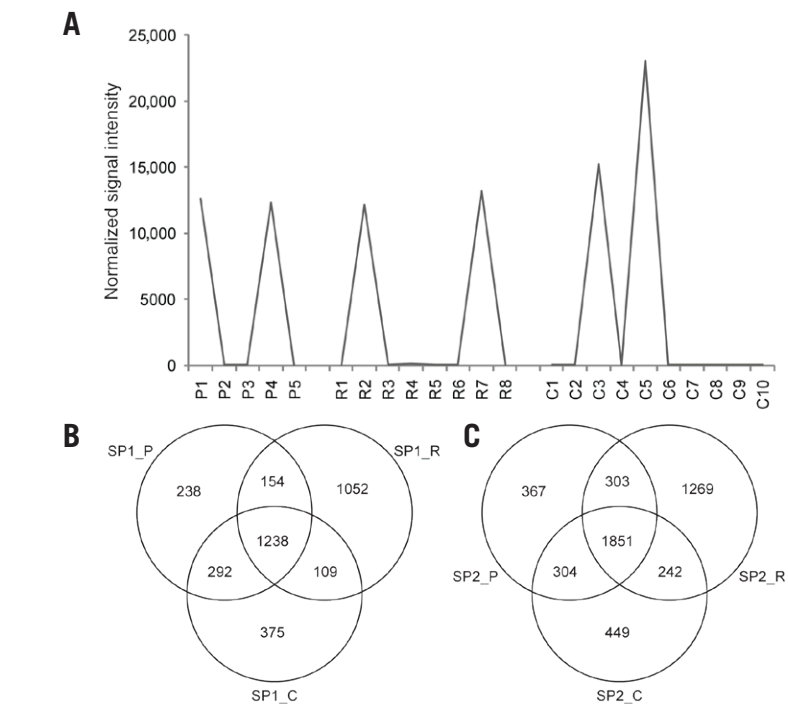


Figure 2. Microarray signal detection and deconvolution. (A) Example of signal patterns from plate (P1–P5), row (R1–R8), and column (C1–C10) matrix pools (y axis indicates normalized signal intensity). Positive signals in this example are from P1, P4, R2, R7, C3, and C5. (B and C) Venn diagram of overlaps between single unigenes from deconvoluted single-hit BACs of SP1 (B) and SP2 (C).

trimmed with a quality score cutoff of 20 to remove poor-quality sequence at the start and end of reads. This left a total of 66,827,380 reads of average length of 62 nucleotides. The combined trimmed reads from all samples were mapped to the Harvest 35 unigene reference sequences using both MOSAIK and SOAPAligner (9) read-mapping tools, in order to be able to compare different outcomes and to eliminate tool-based bias. For each of the mapping programs, two mismatches were allowed.

Validation using quantitative PCR

Assays were designed using the online software of the Universal Probe Library (www.roche-applied-science.com/sis/rtpcr/upl/index.jsp; Roche, Basel, Switzerland) (Supplementary Table S4). Quantitative PCR reactions of BAC-pooled DNA with SYBR Green I were performed on 7500 Fast equipment (Applied Biosystems, Foster City, CA, USA). A total reaction volume of 10 µL included 2 µL unamplified BAC pool DNA (10 ng), 5 µL SYBR Green I PCR Master mix (Sigma-

Table 1. Summary of microarray-based BAC pool screening.

Super pool	Unigenes (single)	Unigenes (double)	Unigenes (total)	Unique BACs (single)	Unique BACs (double)	Unique BACs (total)	Genetic mapping annotation			
							BOPA	Agilent eQTL	Affy eQTL	Unique map positions
SP1	1238	33	1271	531	64	595	82	123	227	155
SP2	1851	15	1866	833	26	859	141	176	363	197
Total	3089	48	3137	1364	90	1454	223	299	590	352

Numbers of probes (unigenes) and single/double hit BACs identified by microarray addressing and their associated genetic map locations derived from Illumina Goldengate- (BOPA)^a and eQTL- (Agilent^b and Affymetrix^c) based mapping.

^aClose, T.J., P.R. Bhat, S. Lonardi, Y. Wu, N. Rostoks, L. Ramsay, A. Druka, N. Stein, et al. 2009. Development and implementation of high-throughput SNP genotyping in barley. *BMC Genomics* 10:582.

^bChen, X., C.A. Hackett, R.E. Niks, P.E. Hedley, C. Booth, A. Druka, T.C. Marcel, A. Vels, et al. 2010. An eQTL analysis of partial resistance to Puccinia hordei in barley. *PLoS One* 5:e8598.

^cPotokina, E., A. Druka, Z. Luo, R. Wise, R. Waugh, and M. Kearsey. 2008. Gene expression quantitative trait locus analysis of 16 000 barley genes reveals a complex pattern of genome-wide transcriptional regulation. *Plant J.* 53:90-101.

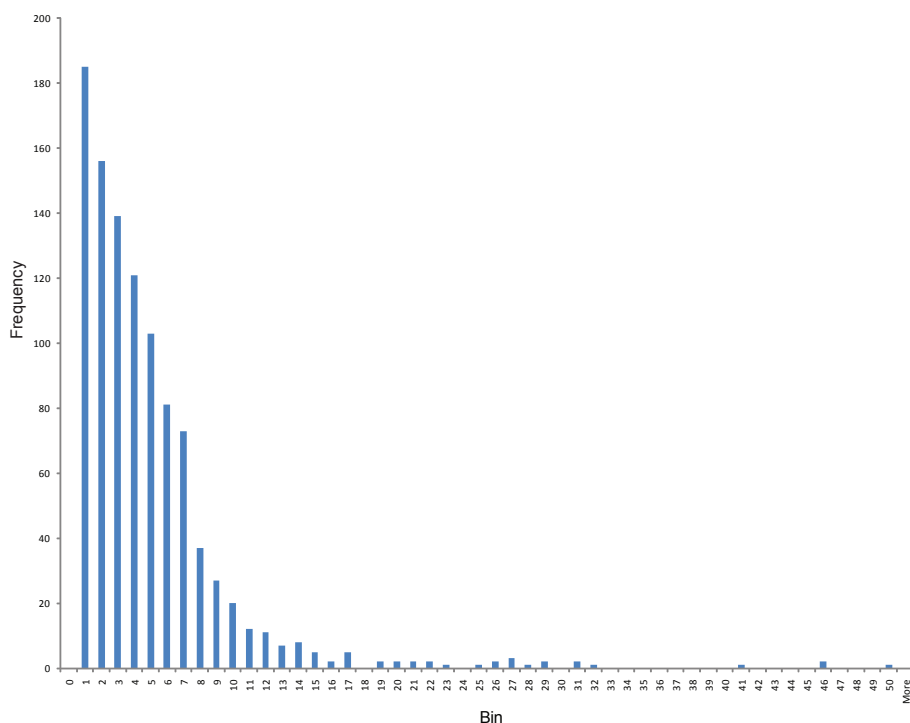


Figure 3. Histogram of frequencies (bin = 1) of nucleotide coverage of mapped 2GS Illumina reads to microarray-positive unigenes. DNA from a single amplified SuperPool (SP1) was subjected to Illumina sequencing, resulting in over 9 million reads. A pronounced bias toward low representation of mapped reads can be seen, with an average coverage of only 4.4x. However, ~87% of microarray-positive unigenes were validated by sequencing.

Aldrich, Dorset, UK) and gene-specific primers (500 nM each). The PCR program was performed as follows: 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Following amplification, a reaction product melting curve analysis was performed (95°C for 15 s, 58°C for 1 min and 95°C for 15 s) to provide evidence for a single reaction product.

Results and discussion

We designed and used an Agilent custom barley array (ArrayExpress: A-MEXP-1728), representing 42,302 expressed genes, to screen whole genome–amplified DNA derived from multidimensional pools of BAC clones that were also incorporated into the HICF physical map (data not shown). The barley BAC library consists of 147,840 clones which were sequentially assigned to 55 Super Pools (SPs) with seven 384-well plates per SP. Plate, row, and column pools from each SP were further pooled, respectively, into five Matrix Plate Pools (MPPs), eight Matrix Row Pools (MRPs), and 10 Matrix Column Pools (MCPs), for a total of 23 Matrix Pools (MPs) (see “Barley BAC library pooling strategy” in the Materials and methods). Each SP is estimated to represent ~1/16th of the

whole barley genome, a feature that reduces the problem of multiple addressing when deconvoluting gene to BAC associations. To date, we have screened a 1x equivalent of the barley genome (i.e., the MPs from 16 SPs; data not shown) to couple gene-to-BAC addresses as a contribution to the International Barley Genome Sequencing Consortium effort (10). We describe here the proof-of-principle involving two SPs: SP1 and SP2.

We independently labeled each MP of SP1 with Cy3 and each MP of SP2 with Cy5, and then co-hybridized each of the 23 pairs of labeled MPs (one MP from each SP) to one of our custom barley microarrays. Therefore, 23 microarrays in total were used to screen all the MPs of both SPs. Following signal extraction from scanned images, data were imported into GeneSpring software (Agilent) to normalize overall signal between each MP microarray. Downstream data processing scripts were written in GenStat software (VSN International) (Supplementary Material) to create a two-stage semi-automated procedure for the identification of BAC clones containing genes corresponding to probes on the microarray. Both stages exploit features of the MP strategy. The first identifies, separately for the plate, row, and column MPs, those probes which have high signals

only two times in a single dimension (Figure 2A). The second combines this information across dimensions (plates, rows, and columns) and matches the output with the known BAC-pooling patterns, to allow deconvolution and identification of the individual BAC clones that contain genes corresponding to probes on the microarray (Figure 2, B and C). The scripts can simultaneously deconvolute single and double BAC hits (the latter have two addresses in one of the three dimensions due to high signals in three MPs for that dimension), with the following criteria: (i) the minimum gScore (weighted log-ratio) of plate, row, and column MPs must be >0.9 for both single and double BAC hits; (ii) for a single BAC hit, the third-highest signal in plate, row, and column MPs must be <100; for double BAC hits, the fourth highest signal in plate, row, and column MPs must be <100. If any of these criteria was not met, the probe was filtered out. After filtering, a total of 1364 BACs (531 from SP1, 833 from SP2) hybridized to 3089 microarray probes (Table 1). This included 738 BACs that hybridized to two or more probes and 626 BACs with a unique hit. Of 74 probes that had double BAC hits, 26 were adjacent in the stock plates, suggesting contamination during processing, and were therefore removed. From the annotation of the positive probes, genetic map locations were available for 25% of the corresponding unigenes, providing genetic anchoring information for 352 BACs from SP1 and SP2 (Table 1; Supplementary Table S1).

In order to validate the microarray hybridization results, four approaches were taken. Firstly, we exploited the strong syntenic relationship between barley and the fully sequenced model cereal *Brachypodium* (11). We examined the 606 of the 738 BACs that hybridized to two or more probes that also had significant homology to *Brachypodium* genes. For 172 of these BACs, multiple probes matched single *Brachypodium* genes reflecting known redundancy on the microarray, thereby providing independent cross validation of the microarray hybridization results. For 434 BACs, the barley probes matched multiple *Brachypodium* genes. Consistent with expected levels of conservation of synteny among grass genomes, 200 (46.1%) BACs hybridized to probes matching adjacent *Brachypodium* genes, thereby providing further validation, and 234 (53.9%) matched nonadjacent genes (Supplementary Table S2).

We then looked for the corresponding barley unigenes in 28 positively hybridizing BAC clones from SP1 that are both present in the emerging barley HICF

BAC-based physical map and have been partially sequenced (data not shown) as part of the International Barley Genome Sequencing Consortium (10) effort. All 28 overlapping BAC sequences contained at least one unigene represented by the corresponding microarray probe (Supplementary Table S3).

We also shotgun-sequenced amplified DNA from SP1 using Illumina 2GS to obtain an open appraisal of its gene content, obtaining 4.5 Gb of total reads, theoretically representing $\sim 12.5\times$ coverage of the total DNA within BAC SP1. Using MOSAIK read-mapping software, 777,945 (1.2%) reads were successfully mapped (≤ 2 mismatches) to 5,728 of the 42,295 unigenes on the microarray. The mapped unigenes included 1017 (82.1%) of the 1238 unigenes anchored by the microarray approach to SP1 BACs. A complementary approach using SOAPAligner read-mapping software identified 86.9% overlap. The smaller number of genes identified by array hybridization can be attributed to the effect of mismatches on probe hybridization kinetics (12), and also incomplete addressing in microarray hybridization, where a single failure out of the six predicted signal patterns will preclude deconvolution

(Supplementary Table S1). That not all microarray positives were detected by 2GS was initially surprising; however, visual examination of the 2GS reads aligned against the reference HarvEST35 dataset revealed highly variable sequence coverage both within and among unigenes. Average nucleotide coverage of only $4.4\times$ was found for the array positive unigenes (compared to $12.5\times$ overall), with pronounced bias towards low representation (Figure 3). The imperfect correspondence is therefore a consequence of the variable 2GS coverage obtained which presumably resulted from inherent technical limitations in sample preparation and data acquisition.

Finally, we used qPCR to test the microarray results by designing gene-specific assays for 55 randomly selected unigenes with gScores ranging 0.9–11.2. These were used to assay the 23 MPs of SP1, and the addresses were deconvoluted and compared to the BACs identified by microarray hybridization. Of the 55 unigenes tested, eight assays failed giving no measurable PCR product in either control cv. Morex genomic DNA or BAC pool DNAs. We attribute these failures to either the presence of partial gene sequences or introns in the genomic DNA,

or SNPs in the primer binding sites when compared to the consensus EST used for assay design. However, of the successful assays, all 47 confirmed the hybridization results (Supplementary Table S1).

We conclude that the method we describe offers an extremely high-throughput, accurate, and cost-effective alternative to current gene-to-BAC addressing methods. This can be extended directly to gene-to-BAC-to-genome addressing if the unigenes represented on the array have also been genetically mapped. The data processing scripts can rapidly deconvolute thousands of addresses and can be adapted to any BAC-pooling strategy with minor modification. The presence of multiple gene-to-BAC addresses within given HICF-based physical contigs will facilitate anchoring, orientating, and ordering genetic and physical maps and providing robust 'homology bridges' to model genomes. The approach can be used in any species that lacks a full genome sequence and paves the way for the generation of genetically anchored physical maps that will find significant and timely application in both forward genetics and reference genome sequencing.

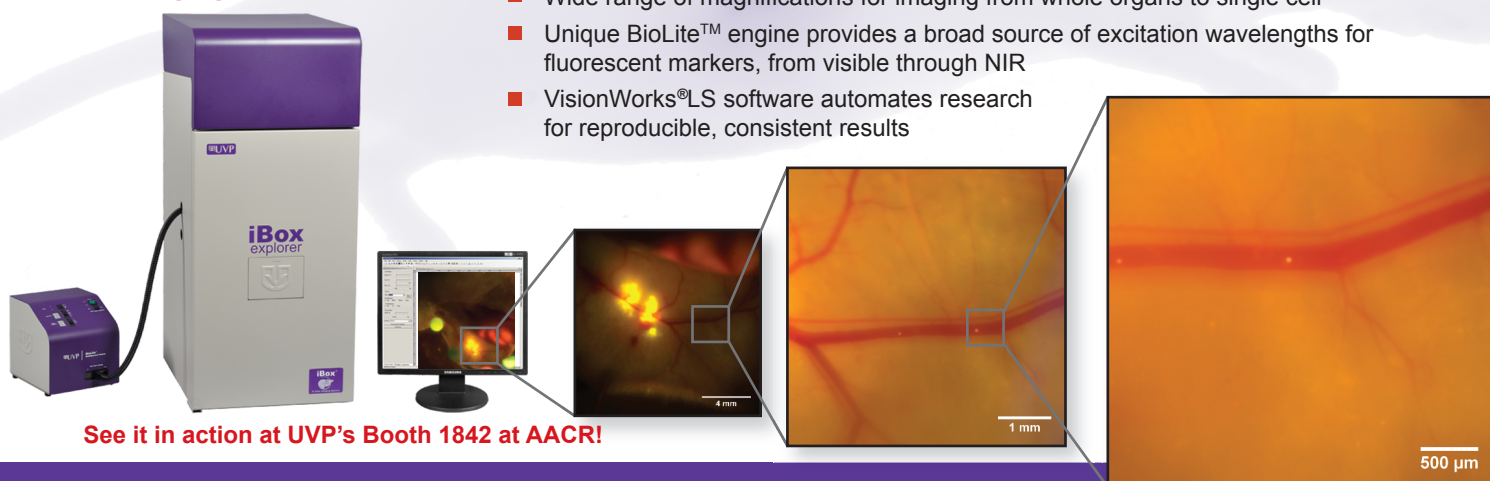
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Competing interests

The authors declare no competing interests.

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